ANTIOXIDANT RESPONSE OF *ARABIDOPSIS THALIANA* TO ZnSe-NANOPARTIPLES, SELENIUM AND ZINC IONS

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Abstract: The impact of zinc selenide nanoparticles (ZnSe-NPs) on plants is still unknown. The intention of this work was to compare phytotoxicity of ZnSe-NPs and selenium and zinc ions in 100, 250 and 500 µM concentrations. Young seedlings of *Arabidopsis thaliana* (Columbia (Col-0) ecotype) was used as an ecotoxicological model. 250 and 500 µM concentrations were extremely phytotoxic and inhibited the growth. Only the lowest concentrations were used for next analysis. ZnSe-NPs treatment had no visible impact on the growth but led to increased antioxidant response. More antioxidant related genes were upregulated than suppressed. Concurrently, there were higher productions of secondary metabolites which are often synthesis during abiotic stress.

Key words: Arabidopsis thaliana, nanoparticles, phytotoxicity, selenium, zinc

INTRODUCTION

Abiotic stress factors have a negative impact on plant growth, yields and quality of plant's products. One of the biggest global problems is heavy metals (Gielen et al. 2016, Tang et al. 2014). However during the last decades, plants have come into contact with a new stress factor – nanoparticles. Nanotechnology is one of the fastest growing industries. NPs have gained considerable importance because of wide variety of applications - in biomedical, optical, and electronic fields (Rico et al. 2013, Kaveh et al. 2013). Due to its large production and use, their release into the environment is inevitable (Ma et al. 2014). They have difficult predictable mechanisms of toxicity compared with ions. Their phytotoxicity depends on the type of nanomaterial, particle size, specific surface area, concentration and the plants (Ma et al. 2014). NPs have a broad effects resulting from interaction with the plant (Ma et al. 2014, Gielen et al. 2016, Tang et al. 2014, Kaveh et al. 2013). Both positive and negative effects have been presented in the literature (Landa et al. 2012, Jia et al. 1999, Lopez-Moreno et al. 2010, Kumar et al. 2013). Kumar et al. (2013) found that 24 nm size gold NPs at 10 and 80 µg/ml concentrations have significantly induced growth and yield enhancement in *Arabidopsis thaliana*. Landa et al. (2012) pointed to potential environmental risks of ZnO on plants (Landa et al. 2012). TiO_2 NPs negatively affected water transport and transpiration in corn plants (Asli and Neumann, 2009). Many studies have been done on NPs phytotoxicity but some areas are still unclear. ZnSe-NPs have become more popular due to semiconductor properties. They have many potential applications (Zhu et al. 2000). But their plant's toxicity is still unstudied. The objective of this work was to compare phytotoxicity of ZnSe-NPs and selenium and zinc ions in young seedlings of *Arabidopsis thaliana (Columbia (Col-0) ecotype)*. Attention was focused on the antioxidant response, including the biosynthesis of the antioxidant secondary metabolites and their associated enzymes.

MATERIAL AND METHODS

SeZn-NPs and medium

The impact of SeZn-NPs, selenium and zinc ions were tested by cultivating *Arabidopsis thaliana* in the presence of 100, 250 and 500 µM SeZn-NPs and zinc or 10, 25 and 50 µM selenium added to the nutrient medium. Zinc was used in the form zinc sulfate heptahydrate, selenium as a sodium selenite. SeZn-NPs were synthesized by the same method reported previously by Moulick et al. 2015 (Moulick et al. 2015) . The germination media was constituted by standard 0.5 strength Murashige and Skoog (MS) salt supplemented with vitamins and solidified with 0.7% agar. The pH of media was adjusted to 5.7 with 1 M sodium hydroxide. SeZn-NPs solution or sterilized stock solutions of selenium or zinc were added to sterilized media at a final concentration. Germination medium without NPs was used as control.

Plant Species and Culture Condition

The seeds of *Arabidopsis thaliana*, ecotype Columbia, were rinsed with 50% ethanol, followed by sterilization in 50% SAVO, and washing in autoclaved distilled water at least three times. Seeds were germinated under sterile conditions 48 hours at 4 °C. Sterilized seeds were incubated in the cultivated plate at 23 °C and illuminated at 130 µmol m^2/s. with a 16 h light/8 h dark photoperiod. After 4 weeks, plants were removed from the medium immersed in liquid nitrogen and stored at -80 °C until the next processing.

Preparation of methanol extracts

The plants were divided into two parts; roots and leafs. The weighing of fresh tissue was homogenized in 1 ml 80% methanol. Then the samples were centrifuged at 12,000 × g for 10 min at room temperature.

Estimation of total antioxidant capacity

The total antioxidant capacity of extracts was investigated by phosphomolybdenum assay, according to the methods (Alam et al. 2013, Kumaran and Karunakaran, 2007). 100 µl of 50 µg/mL extract was dissolved in 1 ml of the reagent solution (0.6 mol/l sulphuric acid, 28 mol/l sodium phosphate and 4 mmol/l ammonium molybdate solutions), and then the mixture was incubated for 90 min at 95 °C. Rutin was used as the standard. When the samples were cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank, which contained the reagent solution and solvent. The total antioxidant capacity was expressed as the equivalent to millimol of trolox per 1 g of fresh weight (mmol trolox/1g FW).

Determination of polyphenols

The total phenolic contents was detected by FC assay, based on the reduction of a phosphowolframate–phosphomolybdate complex by phenolic compounds (Vinson et al. 1998). 100 µl of the extract samples (or standard) and 50 µl of Folin reagent were mixed with 600 µl of water. After reaction for 1 min, 150 µl of 20% sodium carbonate solution and 190 µl of water were added to complete 1ml volume. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature and darkness. Gallic acid was used as the standard.

Determination of flavonoids

The flavonoids were determined by colorimetric method (Jia et al. 1999). 100 µl of the extracts was mixed with 400 µl of water and 30 µl 5% sodium nitrite solution. After 5 min reaction, 30 µl of 10% aluminium chloride hexahydrate was added to the mixture. Subsequent to 5 min, the addition of 200 µl sodium hydroxide was followed. In 15 min, the absorbance of mixture was measured at 510 nm. Quercetin was used as the standard.

RNA isolation

RNA was isolated from roots and leafs of *Arabidopsis thaliana*. Isolation was carried out using PureLink Plant RNA Reagent (Ambion). After isolation, the RNA samples were purified by DNAasa I RNAasa-free (BioLabs). The quality and quantity of isolated RNA samples were measured using Nanodrop. Their integrity was visually assessed on ethidium bromide-stained agarose gels.

Gene expression analysis

Isolated total RNA was the first converted to cDNA using Transcription First Strand cDNA Synthesis Kit (Roche). For PCR reactions, diluted cDNA products were used as template. Specific primers were selected from NCIB verified from BLAST and STAIR database. After that, they were delivered from Sigma-Aldrich. A complete list of primer sequences is provided in Table 1. qPCR assay was performed using Kapa Sybr Fast qPCR (KapaBiosystems). The qPCR amplification programme was 95 °C for 3 min; 95 °C for 15 s, 60 °C for 45 s, repeating 40 cycles, 95 °C for 15 s, 60 °C for 45 s, melting curve for 20 min; and 95°C for 15s. The total volume from the qRT-PCR was 20 µL. Actin 2 was used as a housekeeping gene for normalization. Relative quantity ($2^{-\Delta\Delta Ct}$ method) was then used to calculate relative gene expression level (Livak and Schmittgen, 2001).

Table 1 Overview of selected primers for qPCR analysis.

Locus	Genes	Forward primer (5'-3')	Reverse primer (5'-3')
AT3G18780	*ACT2*	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
AT1G07890	*APX1*	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
AT4G23100	*GSH 1*	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
AT5G27380	*GSH 2*	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC
At2g37040	*PAL*	GCAGTGCTACCGAAAGAAGTG	CGACCTACATTCCTTGATCCTG
At5g13930	*CHS*	CGCATCACCAACAGTGAACAC	TCCTCCGTCAGATGCATGTG

Legend: ACT 2 – Actin 2, APX 1 - Ascorbate peroxidase 1, GSH 1 – γ-Glutamylcysteine synthetase, GSH 2 – Glutathione synthetase, PAL – Phenylalanine ammonia lyase, CHS – Chalcone synthase

Statistical analysis

The data were statistically analysed by one-way ANOVA variance test with subsequent Tukey's comparison test using software R, version 3.4.0 for windows (www.r project.org).

RESULTS AND DISCUSSION

Effect of toxicant on the growth

Growth was suppressed by an increasing concentration of the toxicant. Concentration of 500 µM SeZn-NPs, 500 µM Zn, 50 µM Se and the combination of 500 µM Zn with 50 µM Se were extremely phytotoxic and absolutely inhibited the growth. Concentration of 250 µM SeZn-NPs, 250 µM Zn, 25 µM Se and the combination of 250 µM Zn with 25 µM also highly reduced the growth (Figure 1). Only the lowest concentrations were used for next analysis.

Figure 1 Influence of ZnSe-NPs treatments on the growth of Arabidopsis thaliana

Antioxidant response to toxicant's treatment

NPs as heavy metals are generally considered to be phytotoxicity because of producing reactive oxygen species. The main defence antioxidant mechanism is formed by secondary metabolites such as glutathione (GSH), phytochelatins, ascorbate, phenols, flavonoids, tocopherols, carotenoids and their associated enzymes (Sharma and Dietz, 2009). The evaluating of antioxidant response

was done by gene expression analysis and spectrophotometric analysis of total polyphenols, flavonoids, and total antioxidant activity.

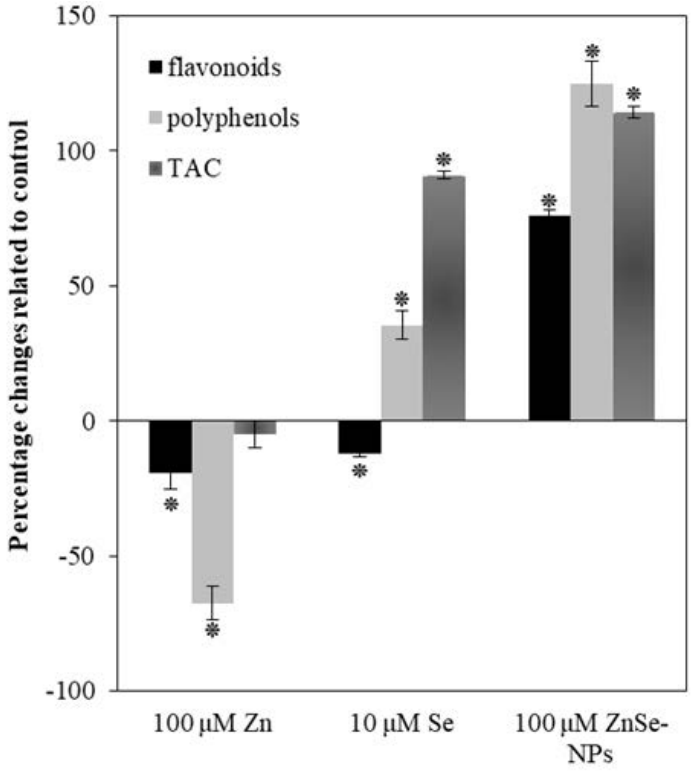
PAL is vital enzymes catalyses reaction of converting L-phenylalanine to ammonia and trans-cinnamic acid that result in thousands of polyphenols. Increased expression of gene coding PAL was manifested in the situation of 100 µM ZnSe-NPs and 10 µM selenium treatments (Figure 2). Simultaneously in these cases, it was established the higher amount of polyphenols compared to control (Figure 3). The total amount of polyphenols in the control samples was 51 mg GAE/g FW. Amount of polyphenols was expressed as the equivalent to milligrams of Gallic acid per 1 g of fresh weight (mmol GAE/1g FW).

Figure 2 Overview of relative expressions of antioxidant related genes

	100 µM ZnSe-NPs	100 µM zinc	10 µM selenium
GSH 1	increased expression ↑↑	increased expression * ↑	increased expression ↑↑ *
GSH 2	increased expression ↑↑	increased expression * ↑	decrease expression
PAL	increased expression ↑↑↑	decrease expression	increased expression ↑
CHS	increased expression ↑↑↑	decrease expression	decrease expression
APX 1	increased expression ↑↑	decrease expression	increased expression ↑

Legend: The relative gene expression level (2−ΔΔCt method for quantitation): ↑ *relative expression 1–3*, ↑ *relative expression 3–5*, ↑ *relative expression 5–>; APX 1 - Ascorbate peroxidase 1, GSH 1 - γ-Glutamylcysteine synthetase, GSH 2 - Glutathione synthetase, PAL - Phenylalanine ammonia lyase, CHS - Chalcone synthase. Stars (*) indicate significant differences compared to the control (p <0.05), (n=3).*

Figure 3 Percentage changes in amount flavonoids, polyphenols and TAC (total antioxidant capacity) related to control. Error bars correspond to standard error of mean. (*) indicate significant differences compared to the control (p <0.05, n=3) according to one-way ANOVA test with subsequent Tukey's comparison test.



Chalcone synthase (CHS) is responsible for synthesis of flavonoids. Except 100 µM zinc treatment, their transcriptomic response to toxicant was higher (Figure 2). This upregulation was followed by increased synthesis of flavonoids (Figure 2). The total amount of polyphenols in the control samples was 4.3 mg RE/g FW. Amount of polyphenols was expressed as the equivalent to milligrams of Gallic acid per 1 g of fresh weight (mmol GAE/1g FW). The exposure to NPs and heavy ions generally induce the biosynthesis of polyphenols and flavonoids involved in the response to abiotic stress (Landa et al. 2012, Moulick et al. 2015, Ma et al. 2013)

Ascorbate peroxidase 1 (APX 1) is part of ascorbate glutathione cycle and belong between the key enzymes to remove H_2O_2 (Panchuk et al. 2005). Higher gene expression for APX 1 was in the samples treated by 100 µM ZnSe-NPs and 10 µM selenium (Figure 1).

Glutathione (GSH) is important molecule that prevents the cell from the oxidative stress. There are two enzymes which are necessary for its biosynthesis (Ma et al. 2013). Except 10 µM selenium in the case of GSH 2, genes were upregulated (Table 1). Total antioxidant activity was higher in every treatment related to control (Figure 2). Total antioxidant activity was expressed as the equivalent to mmol/l of Trolox per 1 g of fresh weight (mmol/l trolox/1g FW). TAC of control samples was 6 mmol/l Trolox/1g FW.

CONCLUSION

Growth was suppressed by an increasing concentration of the toxicant. 250 and 500 µM concentrations were extremely phytotoxic and inhibited the growth. More antioxidant related genes were upregulated than suppressed. Concurrently, there were higher productions of secondary metabolites which are often synthesis during abiotic stress. The strongest antioxidant response and the highest biosynthesis of antioxidant molecules have been found in 100 µM ZnSe-NPs treatment. The weakest antioxidant response was found in 100 µM zinc treatment. The objective of this first experiment was to find out the border of toxicity. The further research will be focus on plant response more deeply and other concentration options will be selected.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grand Agency of Mendel University in Brno IP 42/2017 and CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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